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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/54, C07K 14/745, C12N 1/21, 5/10, A61K 38/36, C12P 1/00, C12N 9/10

A1

(11) International Publication Number: WO 96/21025

(43) International Publication Date: 11 July 1996 (11.07.96)

(21) International Application Number: PCT/US95/17026

(22) International Filing Date: 27 December 1995 (27.12.95)

(30) Priority Data:
08/367,488 30 December 1994 (30.12.94) US

(71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventor: LOK, Si; 806 NW 52nd Street, Seattle, WA 98107 (US).

(74) Agent: SAWISLAK, Deborah, A.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BOVINE FACTOR XIII

(57) Abstract

The present invention provides bovine Factor XIII proteins, DNA molecules and cultured cells expressing bovine Factor XIII. The present invention also provides methods for producing bovine Factor XIII. The invention also includes methods of increasing the water binding capacity of proteins and food products using bovine Factor XIII. Also provided are methods for modifying the amino acid composition of a protein and methods of binding a protein to another insoluble protein using bovine Factor XIII.

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Description

BOVINE FACTOR XIII

Background of the Invention

Factor XIII (also known as plasma transglutaminase) is one of the components of the blood coagulation system, and circulates in the blood in zymogen form until it is activated by thrombin in the final stages of blood coagulation. Activated Factor XIII catalyses the crosslinking of fibrin polymers by introducing covalent bonds between non-covalent fibrin polymers. More specifically, activated Factor XIII catalyses the formation of covalent bonds between free ε-NH^2-lysine groups and γ-glutamic amide bonds in the fibrin polymer. This crosslinking reaction requires the presence of calcium ions (Lorand et al., Prog. Hemost. Thromb. 5: 245-290, 1980). Activated Factor XIII is also known to catalyze crosslinking reactions between other protein molecules, e.g. collagen and fibronectin (Sakata and Aoki, J. Clin. Invest. 65: 290-297, 1980; Mosher J. Biol. Chem. 250: 6614-6621, 1975; Mosher et al. and Chad, J. Clin. Invest. 64: 781-787, 1979; Folk and et al. Adv. Prot. Chem 31: 1-133, 1977; Lorand et al., Prog. Hemost. Thromb. 5: 245-290, 1980).

In placenta, platelets and other cellular sources, Factor XIII exists as an a_2 homodimer (Schwartz et al. J. Biol. Chem. 246:5851-5854, 1971), and in the blood, Factor XIII circulates as a tetrameric complex consisting of two a subunits (Mr of about 83 kDa) containing the catalytic site of the enzyme and two b subunits (Mr of about 80 kDa) (Chung et al., J. Biol. Chem. 249: 940-950, 1974). On activation by thrombin and in the presence of Ca^{++}, the b subunits are cleaved off. Activated Factor XIII is designated as Factor XIIIa. Furthermore, a 4kDa

fragment is cleaved off the N-terminal end of each of the a subunits (Schwartz et al., J. Biol. Chem. 248: 1395-1407, 1973). The potential catalytic site is located in the a chain with cysteine at the active center.

Due to its function in the coagulation process, Factor XIII has been used for treating patients with postoperative wound healing disorders (Mishima et al,. Chirurg. 55: 803-808, 1984) and scleroderma (Delbarre et al., Lancet 2: 204, 1981). Furthermore, Factor XIII has been used as a component of tissue adhesives (U.S. Patent No. 4,414,976; U.S. Patent No. 4,453,939; U.S. Patent No. 4,377,572; U.S. Patent No. 4,362,567; U.S. Patent No. 4,298,598) and has been suggested for use in antifibrinolytic therapy for the prevention of postoperative bleeding and in the treatment of subarachnoid hemorrhage, ulcerative colitis and general wound healing.

Apart from these medical uses, Factor XIII and other transglutaminases have also been proposed for a variety of industrial purposes, primarily within the food industry. The demand for high-quality food proteins and improvement in the functional properties of food proteins is increasing. However, while chemical modifications have been explored, concerns of safety and nutritional effects have prevented their use. The use of enzymatic modification avoids these issues and is therefore considered more appealing for manipulation of food protein. For example, transglutaminase has been added to minced meat and fish paste (see, for example, JP 2-255060 to Ajinomoto, JP 2-227057 to Taiyo Fishery, JP 2-177863 to Ajinomoto) and to milk for the production of cheese (see, for example, JP 2-131537 to Ajinomoto). Transglutaminase has been added to gelatin to make highly polymerized gelatin products (see, for example, JP 2-86743 to Ajinomoto).

In the formulation of food products the use of non-human sources of Factor XIII is preferred. Isolation of such proteins has been an arduous process, as plasma was the common source for non-human Factor XIII. The present invention advantageously provides for the production of recombinant bovine Factor XIII.

Summary of the Invention

The present invention provides recombinant bovine Factor XIII and methods for using bovine Factor XIII. In one aspect, the invention provides a DNA molecule encoding bovine Factor XIII selected from the group consisting of (a) DNA molecules comprising a coding sequence as shown in SEQ ID NO: 1 from nucleotide 62 to nucleotide 2257, (b) DNA molecules complementary to (a), (c) allelic variants of (a) and (b), and (d) DNA molecules that encode for the protein of SEQ ID NO: 2.

Within a related aspect, the present invention provides a DNA construct for the expression of bovine Factor XIII, which comprises the operably linked elements of a transcriptional promoter; a DNA molecule selected from the group consisting of (a) DNA molecules comprising a coding sequence as shown in SEQ ID NO: 1 from nucleotide 62 to nucleotide 2257, (b) DNA molecules complementary to (a), (c) allelic variants of (a) and (b), and DNA molecules that encode the protein of SEQ ID NO: 2; and a transcriptional terminator. Within another related aspect, the present invention includes a cultured cell transformed with the DNA construct comprising the operably linked elements of a transcriptional promoter; a DNA molecule selected from the group consisting of (a) DNA molecules comprising a coding sequence as shown in SEQ ID NO: 1 from nucleotide 62 to nucleotide 2257, (b) DNA molecules complementary to (a), (c) allelic variants of (a) and

(b), and DNA molecules that encode the protein of SEQ ID NO: 2; and a transcriptional terminator.

In another aspect, the present invention provides for bovine Factor XIII polypeptides comprising the amino acid sequence of SEQ ID NO: 2 from amino acid residue 2 to amino acid residue 732.

In another aspect, the present invention provides for methods of producing bovine Factor XIII which comprise culturing the cell transformed or transfected with the DNA construct comprising the operably linked elements of a transcriptional promoter; a DNA molecule selected from the group consisting of (a) DNA molecules comprising a coding sequence as shown in SEQ ID NO: 1 from nucleotide 62 to nucleotide 2257, (b) DNA molecules complementary to (a), (c) allelic variants of (a) and (b), and DNA molecules that encode the protein of SEQ ID NO: 2; and a transcriptional terminator, and isolating the Factor XIII from the cells.

Another aspect of the present invention provides methods for increasing the water binding capacity of a protein comprising mixing a protein that contains a substrate with a bovine Factor XIII, wherein the substrate is crosslinkable by Factor XIII, to provide a mixture, and incubating the mixture for a period of time sufficient for the bovine Factor XIII to react with the substrate, thereby increasing the water binding capacity of the protein. In one embodiment, the protein is selected from the group consisting of casein, caseinate, whey protein, soy protein, blood plasma, fish protein, wheat protein, maize protein, egg albumin, rape seed protein and potato protein.

In another aspect of the present invention, methods for producing a food product with increased water binding capacity are provided comprising mixing a food product that contains a substrate with a bovine Factor XIII, wherein the substrate is crosslinkable by

Factor XIII, to provide a mixture, and incubating the mixture for a period of time sufficient for the bovine Factor XIII to react with the substrate, thereby increasing the water binding capacity of the food product. In one embodiment, the food product is selected from the group consisting of milk and meat from beef, pork, poultry or fish. In another embodiment, the food product comprises a mixture of ingredients, wherein one of the ingredients is a protein selected from the group consisting of casein, caseinate, whey protein, soy protein, blood plasma, fish protein, wheat protein, maize protein, egg albumin, rape seed protein and potato protein.

Another aspect of the present invention provides methods for modifying the amino acid composition of a protein comprising mixing a protein containing a substrate with a bovine Factor XIII and an amino acid, wherein the substrate is crosslinkable by a bovine Factor XIII, to provide a mixture, and reacting the mixture for a period of time to covalently bind the amino acid to the protein.

Another aspect of the present invention provides methods of binding a first protein to a surface of an insoluble second protein comprising reacting a first protein and a bovine Factor XIII with a second, insoluble protein comprising a substrate that is crosslinkable by Factor XIII, for a time sufficient to result in a crosslinked complex of the first protein bound to the surface of the second protein.

These and other aspects of the invention will become evident upon reference to the following detailed description and attached drawing.

Brief Description of the Drawings

The figure illustrates plasmid pD74, a yeast expression construct for bovine Factor XIII.

Detailed Description of the Invention

Prior to describing the present invention in detail, it may be helpful to define certain terms used herein:

Allelic variant: An alternative form of a gene that arises through mutation, or an altered polypeptide encoded by the mutated gene. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence.

cDNA: Complementary DNA, prepared by reverse transcription of a messenger RNA template, or a clone or amplified copy of such a molecule. Complementary DNA can be single-stranded or double-stranded.

Expression vector: A DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both. The term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator.

Gene: A segment of chromosomal DNA that encodes a polypeptide chain. A gene includes one or more regions encoding amino acids, which in some cases are interspersed with non-coding "intervening sequences" ("introns"), together with flanking, non-coding regions which provide for transcription of the coding sequence.

Molecules complementary to: Polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

Promoter: The portion of a gene at which RNA polymerase and other transcription factors bind and mRNA synthesis is initiated.

The present invention provides isolated nucleotide sequences of bovine Factor XIII, thereby providing for the expression of bovine Factor XIII polypeptides and fragments thereof. Useful polynucleotide molecules in this regard include mRNA, genomic DNA, cDNA, synthetic DNA and DNA molecules generated by ligation of fragments from different sources. For production of recombinant bovine Factor XIII, DNA molecules lacking introns are preferred for use in most expression systems. By "isolated" it is meant that the molecules are removed from their natural genetic milieu. Thus, the invention provides DNA molecules free of other genes with which they are ordinarily associated. In particular, the molecules are free of extraneous or unwanted coding sequences, and in a form suitable for use within genetically engineered protein production systems. The term isolated bovine Factor XIII polypeptides and fragments is meant to include sequences of amino acids up to entire proteins, which have at least about 90% identity, and preferably at least about 95% or more identity to the amino acid sequences of the bovine Factor XIII of the invention. As will be appreciated by those skilled in the art, the invention also includes those polypeptides having slight variations in amino acid sequences or other properties. Such variations may arise naturally as allelic variations or may be produced by human intervention (e.g., by

mutagenesis of cloned DNA sequences), such as induced point, deletion and insertion mutants.

Nucleic acid sequences encoding bovine Factor XIII as described herein can be cloned from a variety of bovine cell sources that express the enzyme. Preferred sources for bovine Factor XIII include placenta, platelets, neutrophils and monocytes. Useful isolated nucleic acid sequences of the present invention which encode bovine Factor XIII include mRNA, genomic DNA and cDNA. For expression, cDNAs are generally preferred because they lack introns that may interfere with expression.

To obtain bovine Factor XIII clones, a bovine placental tissue cDNA library was prepared and probes were generated from sequences of human Factor XIII using oligonucleotide primers in a polymerase chain reaction ("PCR"; U.S. Patent Nos. 4,683,195, 4,683,202, incorporated herein by reference). The oligonucleotide primer sequences were complementary to the regions 5' and 3' to the coding region of human Factor XIII and are described in Example I. Those skilled in the art will recognize that alternative tissue sources and techniques can be employed.

To obtain the bovine placental Factor XIII clone, an oligo d(T) primed cDNA library can be constructed with poly A^+ RNA purified from bovine placental tissues. If necessary, partial clones may be used as probes in additional screening until the complete coding sequence is obtained. Joining is achieved by digesting clones with appropriate restriction endonucleases and joining the fragments enzymatically in the proper orientation. Depending on the fragments and the particular restriction endonucleases chosen, it may be necessary to remove unwanted DNA sequences through a "loop out" process of deletion mutagenesis or through a combination of restriction endonuclease cleavage and mutagenesis. It

is preferred that the resultant sequence be in the form of a continuous open reading frame, that is, that it lack intervening sequences (introns). The sequence of an exemplary bovine Factor XIII clone, described herein, includes 2196 nucleotides of coding sequence for the a subunit and is shown in SEQ ID NO: 1, with an open reading from nucleotide 62 to nucleotide 2257.

The present invention also provides isolated Factor XIII polypeptides. In a preferred form the isolated polypeptides is substantially free of other proteins of bovine origin. The exemplary bovine Factor XIII clone described herein is 732 amino acid residues and is shown in SEQ ID NO: 2.

For expression, a DNA sequence encoding bovine Factor XIII is inserted into a suitable expression vector, and the resulting DNA construct is used to transform or transfect appropriate host cells for expression. Expression vectors for use in carrying out the present invention will comprise a promoter capable of directing the transcription of a cloned DNA sequence and a transcriptional terminator, operably linked with the sequence encoding the bovine Factor XIII polypeptide so as to produce a continuously transcribable gene sequence which produces sequences in reading frame and is translated to produce a bovine Factor XIII polypeptide.

Host cells for use in practicing the present invention include bacteria, yeast and cultured mammalian cells. Human Factor XIII cDNA clones and production of Factor XIII in recombinant cells has been described by Grundmann et al. (published Australian patent application 69896/87) and Davie et al. (U.S. Patent Application Serial No: 07/174,287; EP 268,772), which are incorporated herein by reference. Particularly preferred host cells for producing recombinant Factor XIII include yeasts, such as bakers' yeast (Saccharomyces cerevisiae) and species of Pichia

and Kluyveromyces. Methods for expressing cloned DNA sequences are well known in the art. Briefly, a DNA sequence encoding Factor XIII is operably linked to a suitable promoter and terminator sequences in a vector compatible with the chosen host cell. The vector is then inserted into the host cell and the resulting recombinant cells are cultured to produce Factor XIII.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (ibid.), Hinnen et al. (Proc. Natl. Acad. Sci. USA 75: 1929-1933, 1978), Yelton et al. (Proc. Natl. Acad. Sci. USA 81: 1740-1747, 1984), and Russell (Nature 301: 167-169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host cell and selectable marker is well within the level of ordinary skill in the art.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76: 1035-1039, 1978), YEp13 (Broach et al., Gene 8: 121-133, 1979), POT1 vectors Kawasaki et al., ibid.). Another suitable selectable marker is the CAT gene, which confers chloramphenicol resistance on yeast cells.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255: 12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1: 419-434, 1982; Kawasaki, U.S. Patent No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.) p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101: 192-201, 1983). In this regard, particularly preferred promoters are the TPI1 promoter (Kawasaki, U.S. Patent No. 4,599,311, 1986) and the ADH2-4^{c} promoter (Russell et al., Nature 304: 652-654,

1983; Irani and Kilgore, U.S. Patent Application Serial No. 07/784,653, which is incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, ibid.).

Additional vectors, promoters and terminators for use in expressing the Factor XIII of the invention in yeast are well known in the art and are reviewed by, for example, Emr, Meth. Enzymol. 185: 231-279, 1990, incorporated herein by reference.

The bovine Factor XIII of the invention may be expressed in Aspergillus spp. (McKnight and Upshall, described in U.S. Patent 4,935,349, which is incorporated herein by reference). Useful promoters include those derived from Aspergillus nidulans glycolytic genes, such as the ADH3 promoter (McKnight et al., EMBO J. 4: 2093-2099, 1985) and the tpiA promoter. An example of a suitable terminator is the ADH3 terminator (McKnight et al., ibid.).

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred cultured mammalian cells for use in the present invention include the COS-1 (ATCC CRL 1650) and BALB/c 3T3 (ATCC CRL 163) cell lines. In addition, a number of other mammalian cell lines may be used within the present invention, including BHK (ATCC CRL 10314), 293 (ATCC CTRL 1573), Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CRL 139), Human lung (ATCC CCL 75.1) Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CCL 29.1), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77: 4216-4220, 1980).

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a

cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., Cell 41: 521-530, 1985), the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1: 854-864, 1981), and the major late promoter from Adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2: 1304-1319, 1982). Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a mouse V_K promoter (Bergman et al., Proc. Natl. Acad. Sci. USA 81: 7041-7045, 1983; Grant et al., Nuc. Acids. Res. 15: 5496, 1987) and mouse V_H promoter (Loh et al., Cell 33: 85-93, 1983). Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the Adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., Nuc. Acids. Res. 9: 3719-3730, 1981). Vectors can also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, Cell 33: 717-728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs. Vectors can be obtained from commercial sources (e.g., Stratagene, La Jolla, CA).

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14: 725, 1978; Corsaro an Pearson, Somatic Cell Genetics 7: 603, 1981; Graham and Van der Eb, Virology 52: 456, 1973), electroporation (Neumann et al., EMBO J. 1: 841-845, 1982), or DEAE-dextran mediated transfection (Asubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY (1987), incorporated herein by reference). To identify cells that have stably integrated the cloned DNA, a

selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred selectable marker is the DHFR gene. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA, which is incorporated herein by reference). The choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate vector at the same time as the Factor XIII sequence of interest, or they may be introduced on the same vector. If on the same vector, the selectable marker and the Factor XIII sequence of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Promoters, terminators and methods for introducing expression vectors encoding Factor XIII into plant, avian and insect cells are well known in

the art. The use of baculoviruses, for example, as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by (Atkinson et al. Pestic. Sci. 28: 215-224, 1990). The use of Agrobacterium rhizogenes as vectors for expressing genes in plant cells has been reviewed by (Sinkar et al. J. Biosci. (Banglaore) 11: 47-58, 1987).

Host cells containing DNA constructs of the present invention are then cultured to produce Factor XIII. The cells are cultured according to standard methods in a culture medium containing nutrients required for growth of the chosen host cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals, as well as other components, e.g., growth factors or serum, that may be required by the particular host cells. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct.

Yeast cells, for example, are preferably cultured in a medium which comprises a nitrogen source (e.g., yeast extract), inorganic salts, vitamins and trace elements. The pH or the medium is preferably maintained at a pH greater than 2 and less than 8, preferably at pH 5-6. Methods for maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, MO). Cultured mammalian cells are generally cultured in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level or ordinary skill in the art.

In a preferred embodiment, the bovine Factor XIII are expressed in yeast as intracellular products. The yeast host cell can be a diploid strain homozygous for pep4, a mutation that reduces vacuolar protease levels, as described in Jones et al., Genetics 85: 23-33, 1977, incorporated herein by reference. The strain is also homozygous for disruption of the endogenous TPI (triose phosphate isomerase) gene, thereby allowing S. pombe POT1 gene to be used as a selectable marker. The vector includes the POT1 marker, a leu2-d marker and ADH2-4^{c} promoter. The POT1 marker in the TPI^{-} host allows for selection by growth in glucose. The host strain is grown in glucose-containing synthetic media with a glucose feed. An ethanol feed is then substituted for glucose to de-repress the promoter. The pH is maintained with NaOH. Other preferred means for expression are generally described in, e.g., EPO publication EP 268,772, incorporated herein by reference.

Depending on the particular host cell and the expression unit utilized, the Factor XIII may either be secreted from the cells or retained in the cytoplasm. When using cells that do not secrete Factor XIII, the cells are removed from the culture medium (e.g., by centrifugation) and treated to produce a lysate. Typically, yeast cells are treated by mechanical disruption using glass beads to produce a crude lysate. Preferably, the crude lysate is centrifuged, and the supernatant fraction is recovered. The supernatant is treated to produce a cleared lysate, typically by centrifugation at moderate speed (e.g., 10,000 x g) or filtration through a high molecular weight cutoff membrane.

When working with crude lysates, which are likely to contain high levels of proteases, it is preferred to minimize the time in which the lysate is in a concentrated form. This can be readily achieved

by quickly diluting the lysate, preferably in cool (2-5°C) water. In general, the lysate will be diluted about 3 to 10-fold relative to the starting cell slurry. Factor XIII may also be obtained from cells that secrete it into the culture medium. Cells are transformed to express Factor XIII subunits with an attached secretory signal sequence, which is removed from the Factor XIII protein by proteolysis as it transits the secretory pathway of the host cell. For purification of the Factor XIII, the cells are removed by centrifugation, the medium is fractionated, and the Factor XIII is recovered.

The Factor XIII product of the invention may conveniently be provided in the form of a Factor XIII a_2 dimer (i.e. placental Factor XIII). The Factor XIII product of the invention is advantageously a recombinant protein since this is a more reliable and economical source of Factor XIII than plasma.

Factor XIII may be activated with an immobilized proteolytic enzyme. Examples of suitable enzymes are thrombin, trypsin or a trypsin-like enzyme (e.g. a protease obtainable from a species of Fusarium, cf. WO 89/06270). The proteolytic enzyme may suitably be immobilized by one of the procedures described in K. Mosbach (ed.), "Immobilized Enzymes" in Methods in Enzymology 44, Academic Press, New York, 1976, including covalent coupling to insoluble organic or inorganic supports, entrapment in gels and adsorption to ion exchange resins or other adsorbent materials. Coating on a particulate support may also be employed (see Macrae et al., Biotechnology and Genetic Engineering Reviews 3: 193, 1985). Suitable support materials for the immobilized enzyme are, for instance, plastics (e.g. polypropylene, polystyrene, polyvinylchloride, polyurethane, latex, nylon, teflon, dacron, polyvinylacetate, polyvinylalcohol or any suitable copolymer thereof), polysaccharides (e.g.

agarose or dextran), ion exchange resins (both cation and anion exchange resins), silicon polymers (e.g. siloxane) or silicates (e.g. glass).

Alternatively, the Factor XIII may be contacted with a proteolytic enzyme after which a protease inhibitor is added. The protease inhibitor may suitably be a trypsin inhibitor such as aprotinin or soybean trypsin inhibitor.

The buffer solution into which the activated Factor XIII is collected is preferably a glycine, alanine or borate buffer.

The stabilizer or stabilizers present in the buffer solution as well as in the final Factor XIII composition may be a chelating agent, for instance EDTA, EGTA or citrate. EDTA may be present in a concentration of 2-15 mM, preferably 3-12 mM, more preferably 5-10 mM. Another stabilizer which may be present in the buffer solution and Factor XIII composition is a reducing agent or another substance capable of preventing oxidation of the active -SH at Cys314 of Factor XIII, e.g. a cysteine or sulfite, or an antioxidant such as ascorbic acid or glutathion. An example of a suitable reducing agent is dithiothreitol (DTT), which may be present in a concentration of 1-10 mM, preferably 2-7 mM, more preferably 2.5-5 mM. A further stabilizer which may be present in the buffer solution and Factor XIII composition is a sugar. Examples of suitable sugars are lactose, glucose, sucrose, maltose or trehalose. The sugar may be present in an amount of 0.5-5%, preferably 1-2%, by weight. A still further stabilizer which may be present in the buffer solution and Factor XIII composition is casein. Incidentally, it should be noted that when the activated Factor XIII of the invention is used for crosslinking reactions, calcium ions should be present.

A preferred stabilizing solution comprises 2% lactose, 2% casein, 10 mM EDTA, and 5 mM DTT in 10 mM glycine buffer, pH 8.0.

The Factor XIII of the present invention can be in freeze-dried form as this generally results in improved stability.

The present invention provides methods for increasing the water binding capacity of a protein that contains a substrate that is crosslinkable by Factor XIII. The protein is reacted with bovine Factor XIII. The reaction mixture is incubated for a period of time sufficient for the bovine Factor XIII to react with the substrate resulting in ε(γglutmyl)lysyl crosslinked polymers. The resulting increase in the water binding capacity will be perceived as an increase in the viscosity and/or increase in the gel strength of the resulting product. Methods for measuring gel strength and viscosity are known in art (see, for example, Klettner, Fleischwirtsh, 69 (2):225-226, 1989; Suzuki T. Fish and Krill Protein: Processing Technology, APII Publishers Ltd. London, 1981; Prentice, J. Measurements in the Rheology of Foodstuffs, Elsevier Applied Science Publisher, London, 1984; Montejano et al. J. Rheology 27 (6):557-579, 1983 and Montejano et al. J. Food Science 49:1496-1505, 1984) and may be made using a rheometer (SunRheotex, Toyko, Japan), an Instron dynamometer (Instron, FGR), a Bloom gelometer (Griffin and George Ltd., Great Britain) or the like.

New protein products with lowered concentrations of protein ingredients can be made by utilizing the increased water binding properties. One such product would be, for example, gelatin that is insoluble at temperatures above 40°C. Any protein that contains free ε-NH_2-lysine and γglutamic amide groups can act as a substrate, that is, crosslinkable by Factor XIII and can be used. The substrate can be found in protein-containing foods like milk and meat.

Preferred are the meat of beef, pork, poultry or fish. The protein can be included as one of the ingredients in a food product such as dessert products, confectionary products and dressings. The substrate can also be found in a concentrated or isolated protein product which is used as an ingredient in another product. Sources of proteins with Factor XIII substrates include animal, plant, yeast or microbial proteins. Preferred proteins include casein, caseinate, whey protein, soy protein, blood plasma, fish protein, wheat protein, maize protein, egg albumin, rape seed protein and potato protein (Traoré et al., J. Agric. Food Chem. 39: 1892-1896, 1991 and Traoré et al., J. Agric. Food Chem. 40: 399-402, 1992; all incorporated herein by reference).

When the methods of the present invention are used to produce a food product with increased water binding capacity, the resulting alteration in the functional properties can be utilized to lower the fat content in the food product because the crosslinked protein simulates properties associated with a higher fat content (Budolfsen et al., WO 93/22930; incorporated herein by reference). The methods can be used in the preparation of restructured meat products, e.g. processed ham, containing finely diced meat or emulsified meat products such as sausages or chopped beef or pork, optionally together with soy protein. The bovine Factor XIII of the present invention may be added to the meat material before, during or after dicing or blending. After incubation, the mixture may be put into appropriate containers, such as sausage casings or tins, and boiled.

Other food products that can be produced using the methods of the present invention include fish paste products with improved consistency properties, production of sausage casings by crosslinking of collagen, in cheesemaking for improving the yield of

cheese by crosslinking soluble whey proteins, in baking for strengthening gluten and in the food industry for making edible protein films for wrapping meat or fish products.

The bovine Factor XIII is added in an amount of 0.001 to 5 parts by weight to 100 parts by weight of the protein. Calcium salts are in an amount of 0.001 parts by weight to 2 parts by weight of the protein or food product to enhance the crosslinking activity of the bovine Factor XIII. The determination of amounts and conditions for adding transglutaminases to food products resulting in increased water binding capacity are known in the art and well within the skill of one skilled in the art. See, for example, U.S. Patent No. 4,917,904, which is incorporated herein by reference.

The methods of the present invention also provide for modifying the amino acid composition of a protein by covalently binding an amino acid to a protein substrate by use of bovine Factor XIII. The amino acid may be an isolated amino acid, a component of an amino acid mixture, or a component of a polypeptide. The amino acids, protein containing the substrate that is crosslinkable by Factor XIII and the bovine Factor XIII are prepared as a mixture and allowed to react for a period of time sufficient to covalently bind the amino acid to the protein. This method could be used, for example, to increase the nutritional value of a protein by binding essential amino acids to the protein.

The present invention also provides methods for binding a first protein to the surface of an insoluble second protein containing a substrate that is crosslinkable by Factor XIII. The first protein, second, insoluble protein and bovine Factor XIII of the present invention are reacted for a period of time sufficient to result in the first protein forming a crosslinked complex with the second protein by binding

to the surface of the second protein. In this way, it is possible to obtain a surface of an item which has an improved appearance or is more resistant, for example, for leather finishing.

The present invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

Example I

Probe preparation

Human placental Factor XIII cDNA was used as a probe to screen for a bovine FXIII cDNA. pD16, a yeast expression vector containing the cDNA sequence encoding the a subunit of human placental Factor XIII (Bishop et al., Biochemistry 29: 1861-1869, 1990) was used as a template for polymerase chain reaction (PCR) amplification of human placental Factor XIII cDNA. The oligonucleotides ZC667 SEQ ID NO: 3 (an 18bp sense primer in the ADH2-4c promoter region of pD16, about 90bp upstream from ATG from human Factor XIII) and ZC2045 SEQ ID NO: 4 (a 17bp antisense primer in TPI terminator region of pD16, about 70 bp downstream from human Factor XIII termination) were used as primers.

Two one-hundred-microliter reactions were set up with each reaction containing 1ng human FXIII cDNA template, 10 µl of 10X PCR Buffer (Promega Corp.), 6 µl of 25mM $MgCl_2$, 1 µl of 20mM deoxynucleotide triphosphate mix containing dCTP, dGTP, dATP and dTTP, 5 µl each of the 20pmol/µl primers and 71 µl water. The reaction mixtures were heated to 80°C in a Perkin-Elmer Cetus DNA thermal cycler at which time 1 µl of 5 U/µl AmpliTaq® DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT.) was added. The reactions were overlayed with mineral oil and amplified for 30 cycles at 95°C

for 1 minute, 30°C for 30 seconds and 72°C for 3 minutes, followed by one cycle at 72°C for 10 minutes.

The reaction mixtures were pooled, and an aliquot of the reaction mix was analyzed by gel electrophoresis on a 0.8% agarose gel. A single 2,361 bp band of the expected size was seen. The pooled mixture was precipitated with 100 µl of 7.5 M ammonium acetate and 2.5 volumes ETOH at -20°C for 18 hours. The DNA was pelleted, resuspended in 100 µl TE, purified on a CHROMOSPIN 400 (CLONTECH Laboratories, Inc., Palo Alto, Ca.) spin column according to the manufacturer's recommendation and precipitated in 2.5 volumes ETOH as described above. The probe DNA was resuspended to a final concentration of 60 ng/µl.

Example II

Synthesis of cDNA and Preparation of cDNA Libraries

A. Bovine Placental cDNA Synthesis

Total RNA was prepared from the bovine placental tissue using guanidine isothiocyanate (Chirgwin et al., Biochemistry 18: 52-94, 1979) and CsC1 centrifugation. Poly(A)$^+$ RNA was isolated using oligo d(T) cellulose chromatography (Aviv and Leder, Proc. Natl. Acad. Sci USA 69: 1408-1412, 1972).

First strand cDNA was synthesized from two-time oligo d(T) selected bovine placenta poly(A)$^+$ RNA. Ten microliters of a solution containing 10 µl of 20 pmole/µl first strand primer ZC6091 (SEQ ID NO: 5), 8.2 µl of 1.6 µg/ml mRNA and 4 µl of diethylpyrocarbonate-treated water. The mixture was heated at 65°C for 4 minutes and cooled by chilling on ice.

The first strand cDNA synthesis was initiated by the addition of 8 µl of 5X SUPERSCRIPT buffer (GIBCO BRL, Gaithersburg, Md.), 4 µl of 100 mM dithiothreitol and 2.0 µl of a deoxynucleotide triphosphate solution

containing 10 mM each of dATP, dGTP, dTTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology Inc,. Piscataway, NJ) to the RNA-primer mixture. The reaction mixture was incubated at 45°C for 3 minutes. After incubation, 10.0 µl of 200 U/µl SUPERSCRIPT reverse transcriptase (GIBCO BRL) was added. The efficiency of the first strand synthesis was analyzed in a parallel reaction by the addition of 10 µCi of ^{32}P-αdCTP to a 5 µl aliquot of the reaction mixture to label the reaction products. The first strand synthesis reaction mixtures were incubated at 45°C for 60 minutes followed by a 15 minute incubation at 50°C. Unincorporated nucleotides were removed from each reaction by twice precipitating the cDNA in the presence of 6 µg of glycogen carrier, 2.5 M ammonium acetate and 2.5 volume ethanol. The unlabeled cDNA was resuspended in 50 µl water and used for the second strand synthesis. The efficiency and length of first strand cDNA synthesis was assessed by analysis the labeled cDNA by agarose gel electrophoresis.

Second strand synthesis was performed on the RNA-DNA hybrid from the first strand synthesis reaction under conditions that promoted first strand priming of second strand synthesis resulting in DNA hairpin formation. A reaction mixture was prepared containing 20.0 µl of 5X polymerase I buffer (100 mM Tris, pH 7.4, 500 mM KCl, 25 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$), 1.0 µl of 100 mM dithiothreitol, 2.0 µl of a solution containing 10 mM of each deoxynucleotide triphosphate, 3.0 µl of β-NAD, 15.0 µl of 3 U/µl *E.coli* DNA ligase (New England Biolabs, Beverly, MA), 5.0 µl of 10 U/µl *E. coli* DNA polymerase I (GIBCO BRL) and 48.0 µl of the unlabeled first strand DNA. A parallel reaction in which a 10 µl aliquot of the second strand synthesis was labeled by the addition 10 µCi of ^{32}P-αdCTP was used to monitor the efficiency of second strand synthesis. The reaction mixtures were incubated at room temperature

for 5 minutes followed by the addition of 1.5 µl of 2 U/µl RNase H (GIBCO BRL) to each reaction mixture. The reactions were incubated at 15°C for 2 hours followed by a 15 minute incubation at room temperature. The reactions were terminated by addition of water to a final volume of 100 µl followed by two phenol/chloroform (1:1) extractions and one chloroform/isoamylalcohol (24:1) extraction. The DNA from each reaction was precipitated in the presence of ethanol and 2.5 M ammonium acetate as described above. The DNA from the unlabeled reaction was resuspended in 100.0 µl water. The labeled DNA was resuspended and electrophoresed as described above.

The single-stranded DNA in the hairpin structure was cleaved using mung bean nuclease. The reaction mixture contained 20.0 µl of 10X Mung Bean Nuclease Buffer (Stratagene Cloning Systems, La Jolla, Calif.), 16.0 µl of 200 mM dithiothreitol, 49.0 µl water, 50.0 µl of the second strand cDNA and 15.0 µl of a 1:10 dilution of Mung Bean nuclease (Promega Corp., Madison, Wis.) in Stratagene MB dilution Buffer (Stratagene Cloning Systems). The reaction was incubated at 37°C for 20 minutes, and the reaction was terminated by the addition of 20.0 µl of 1M Tris-HCl, pH 8.0 followed by sequential phenol/chloroform and chloroform/isoamylalcohol extractions as described above. Following the extractions, the DNA was precipitated in ethanol and resuspended in water.

The resuspended cDNA was blunt-ended with T4 DNA polymerase. The cDNA, which was resuspended in a volume of 140 µl of water, was mixed with 50.0 µl of 5X T4 DNA polymerase buffer (250 mM Tris-HCl, pH 8.0, 250 mM KCl, 25 mM $MgCl_2$), 3.0 µl of 100 mM dithiothreitol, 3.0 µl of a solution containing 10 mM of each deoxynucleotide triphosphate and 4.0 µl of 4 U/µl T4 DNA polymerase (Boehringer Mannheim). After an incubation at 15°C for 30 minutes, the reaction was

terminated by by serial phenol/chloroform and chloroform/isoamylalcohol extractions as described above. The DNA was ethanol precipitated and resuspended in 30.0 µl of water.

B. Preparation of a Bovine Placenta cDNA Library

Eco RI adapters (Pharmacia LKB Biotechnology Inc.) were added to the cDNA prepared above to facilitate the cloning of the cDNA into a mammalian expression vector. A 9.0 µl aliquot of the cDNA and 975 pmole of the adapter (15.0 µl) were mixed with 3.0 µl 10X Promega B ligase buffer (Promega), 4.0 µl 10mM ATP, 6.0 µl water and 30 Weiss Units of Promega DNA ligase (2.0 µl; Promega). The reaction was incubated for 48 hours at 10°C. The reaction was terminated by the addition of 150.0 µl of water, 20.0 µl of 3M sodium acetate followed by an incubation at 65°C for 30 minutes. After incubation, the reaction was phenol/chloroform extracted followed by a chloroform/isoamylalcohol extraction and ethanol precipitation as described above. Following centrifugation, the DNA pellet was washed with 70% ethanol and was air dried. The pellet was resuspended in 88.5 µl of water.

The directional insertion of the cDNA into a mammalian expression vector was achieved by digesting the cDNA with Xho I, resulting in a cDNA having a 5' Eco RI adhesive end and a 3' Xho I adhesive end. The restriction digestion was terminated by serial phenol/chloroform and chloroform/isoamylalcohol extractions. The cDNA was ethanol precipitated, and the resulting pellet was washed with 70% ethanol and air-dried. The pellet was resuspended in 1x loading buffer (10 mM phosphate buffer, pH 8.8, 5% glycerol, 0.125% bromphenol blue).

The resuspended cDNA was heated to 65°C for 10 minutes, cooled on ice and electrophoresed on a 0.9%

low melt agarose gel (Seaplaque® GTG Low Melt Agarose, FMC Corp., Rockland, ME) using the BRL 1 kb ladder (GIBCO BRL) and the Pharmacia 100 bp ladder (Pharmacia LKB Biotechnology Inc.) as size markers. Fragments below 600 bp in size were excised from the gel. The electrodes were reversed and the cDNA was electrophoresed until concentrated near the lane origin. The area of the gel containing the concentrated DNA was excised, placed in a microfuge tube, and the approximate volume of the gel slice was determined. An aliquot of TE equivalent to half the volume of the gel slice was added to the tube, and the agarose was melted by heating to 65°C for fifteen minutes. Following equilibration of the sample to 42° C, 5 units of β-Agarase I (New England Biolabs, Beverly, Mass.) was added. The sample was incubated for 90 minutes to digest the agarose. After incubation, a 0.1 X volume of 3M sodium acetate was added to the sample, and the mixture was incubated on ice for fifteen minutes. After incubation, the sample was centrifuged at 14,000 x g for fifteen minutes at 4° C to remove the undigested agarose. The cDNA in the supernatant was ethanol precipitated. The cDNA pellet was washed with 70% ethanol, air dried and resuspended in 40 µl of water for the kinase reaction to phosphorylate the ligated *Eco* RI adapters.

Five microliters of 10x ligase buffer (Stratagene Cloning Systems) was added to the 40.0 µl cDNA solution described above, and the mixture was heated to 65°C for 5 minutes. The mixture was cooled on ice, and 5.0 µl of 10mM ATP and 3.0 µl of 10U/µl T4 polynucleotide kinase (Stratagene Cloning Systems) were added. The reaction was incubated at 37°C for 45 minutes and was terminated by heating to 65°C for 10 minutes followed by serial extractions with phenol/chloroform and chloroform. The phosphorylated cDNA was ethanol precipitated in the presence of 2.5 M

ammonium acetate, washed with 70% ethanol, air dried and resuspended in 10.0 µl water. The concentration of the phosphorylated cDNA was estimated to be approximately 40 fmole/µl.

The resulting cDNA was cloned into the lambda phage vector λZapII (Stratagene Cloning Systems), that came predigested with *Eco RI* and *Xho* I and dephosphorylated. Ligation of the cDNA to the λZapII vector was carried out in a reaction mixture containing 1.0 µl of 40 fmole/µl prepared vector, 4.5 µl water, 1.0 µl 10x ligase buffer (Promega Corp.), 2.0 µl of 40 fmole/µl cDNA and 1.0 µl of 15U/µl T4 DNA ligase (Promega Corp.). The ligation mixture was incubated at 4°C for 48 hours. Approximately 50% of the ligation mixture was packaged into phage using GIGAPACK II Gold packaging extract (Stratagene Cloning Systems) and the resulting library titered according to the manufacturer's directions, yielding 4.4 x 10^3 plaque forming units(pfu)/µl.

Example III

Isolation of Bovine FXIII cDNA

A primary bovine placenta library was screened for the cDNA encoding bovine Factor XIII using the human Factor XIII cDNA probe described in Example I. The library was titered, and 35 150-mm plates inoculated with *E. coli* SURE® cells (Stratagene Cloning Systems) were infected with 4 x 10^4 pfu. The plates were incubated overnight at 39°C. Filter plaque lifts were made using HYBOND-N™ nylon membranes (Amersham) according to the procedure recommended by the manufacturer. The filters were processed by denaturation in a solution containing 1.5 M NaCl and 0.5 M NaOH for 7 minutes at room temperature. The filters were blotted briefly on filter paper to remove excess denaturation solution followed by neutralization for 5 minutes in 1 M Tris-HCl (pH 7.5) and 1.5 M NaCl.

Phage DNA was fixed onto the filters with 1,200 μJoules of UV energy in a STRATALINKER® UV crosslinker (Stratagene Cloning Systems). After fixing, the filters were prehybridized in hybridization solution (5X SSC, 5X Denhardt's solution, 0.2% SDS and 1 mM EDTA) that had been filtered through a 0.45 μM filter. Heat denatured, sheared salmon sperm DNA (final concentration 100 μg/ml) was added immediately before use. The filters were prehybridized at 65°C overnight.

The human Factor XIII cDNA probe was labeled with ^{32}PdCTP by random priming using the MEGAPRIME™ DNA Labeling System (Amersham) according to the method recommended by the manufacturer. The prehybridization solution was replaced with fresh hybridization solution containing approximately 1.6 x 10^6 cpm probe and allowed to hybridize overnight at 65°C. After hybridization, the hybridization solution was removed, and the filters were rinsed four or five times each in a wash solution containing 0.25x SSC, 0.25% SDS, and 1mM EDTA at room temperature. After rinsing, the filters were washed in eight consecutive washes at 50°C in wash solution. Following the final wash, the filters were exposed to autoradiograph film (XAR-5; Eastman Kodak Co.; Rochester, NY) for one day at -70°C and 2 days at room temperature with an intensifying screen.

Examination of the autoradiographs revealed approximately 35 regions that hybridized with the labeled probe. Agar plugs were picked from 35 regions for purification. Each agar plug was soaked overnight in 1 ml of SM containing 1% (v/v) chloroform (Maniatis et al., eds., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, 1982, incorporated herein by reference). After the overnight incubation, the phage from each plug were diluted 1:1,000 in SM. Aliquots of 12.5 μl were plated on *E. coli* SURE® cells. The plates were incubated overnight at 39°C, and filter

lifts were prepared, prehybridized, hybridized, washed and autoradiographed as described above.

Examination of the resulting autoradiographs revealed positive signals on all but 4 of the 35 filter lifts. Agar plugs were picked from the positive areas for each of the 31 signals. The agar plugs were soaked in 300.0 ml SM and 7.5 ml chloroform, and placed at 4°C overnight. The phage eluted from 20 of the 31 agar plugs were diluted 1:4,000 in SM, and aliquots of 10.0 µl were plated with SURE® cells. The plates were incubated, and phage filter lifts were prepared and hybridized as described above. The filters were washed at 50°C in wash buffer. Autoradiographs of the filters revealed isolated positive signals on all 20 isolates. One plaque was picked from each plate from the tertiary screen, and the small agar plugs were placed in 90.0 µl SM/10 µl chloroform.

The ExAssist™/SOLR system (Stratagene) was used according to manufacturer's specifications to excise Bluescript phagemids from 4 of the 20 plaques described above. The four positives were amplified by PCR for insert size determination. Each PCR reaction contained 1.0 µl template, 0.5 µl of 20mM deoxynucleotide triphosphate mix containing dCTP, dGTP, dATP and dTTP, 2.5 µl of 20 pmol/µl ZC218 (SEQ ID NO. 6), 2.5 µl of 20 pmol/µl ZC219 (SEQ ID NO. 7), 10.0 µl 10X PCR Buffer (Promega Corp.), 6.0 µl 25mM $MgCl_2$, 77.0 µl water and 0.5 µl 5 U/µl AmpliTaq® DNA Polymerase (Perkin-Elmer Cetus). The reaction mixes were overlayed with mineral oil and amplified for 30 cycles using a Perkin-Elmer Cetus DNA thermal cycler at 95°C, 30 seconds (with an additional 1 minute for the first cycle only); 52°C, 30 seconds; and 72°C, 3 minutes; followed by one cycle at 72°C for 10 minutes. An aliquot of each reaction mix was electorphoresed on a 0.8% agarose gel. Two of the clones (#8 and #15) produced bands approximately 4 kb. Clone 5 produced a

band approximately 3.7 kb and clone 6 produced a approximately 2.9 kb band. All clones were sequenced. Clone 15 was shown to contain the sequence shown in SEQ ID NO: 1 and was designated pBF13.

Example IV

Expression of bovine Factor XIII in yeast

A. Assembly of the bovine Factor XIII N-terminus end adapted plasmid, pBglbf13.

pBF13 was digested with Ava I and Eco RI, and a 1.59 kb coding sequence of bovine FXIII was isolated. Ava I cuts 108 bp downstream of the bovine Factor XIII start codon. Oligonucleotides (ZC7386 SEQ ID NO: 8, ZC7396 SEQ ID NO: 9, ZC7389 SEQ ID NO: 10 and ZC7382 SEQ ID NO: 11) were used to reconstruct the bovine cDNA from the start codon to the Ava I site as well as add a Bgl II and Bam HI site immediately 5' to the start codon. The oligonucleotides and the bovine Factor XIII Ava I-Eco RI fragment were subcloned into a Bam HI-Eco RI digested pUC 19 and was designated pBglbf13. The insert was confirmed by sequence analysis. pBglbf13 was digested with Bgl II and Eco RI, and this approximately 1.7 kb fragment, containing the coding sequence of the N-terminus of bovine FXIII along with the Bgl II site 5' to the start codon, was isolated.

B. Assembly of the bovine Factor XIII carboxy-terminus adapted plasmid, ptermbf13.

pBF13 was digested with Eco RI and Afl III, and a 0.45 kb coding sequence of bovine FXIII was isolated. Afl III cuts 46 bp upstream of the bovine Factor XIII stop codon. Oligonucleotides (ZC7384 SEQ ID NO: 12 and ZC7383 SEQ ID NO: 13) were used to reconstruct bovine Factor XIII from the Afl III site to the 3'end, immediately followed by a stop codon and an Xba I site. The bovine Factor XIII Eco RI-Afl III

fragment, the oligonucleotides and an Xba I-Bam HI fragment from pZV244 containing the TPI1 terminator (U.S. Patent 4,931,373) were ligated into Bam HI-Eco RI digested pUC19 to generate plasmid ptermbf13. The insert was confirmed by sequence analysis. ptermbf13 was digested with Eco RI and Sal I, and an approximately 1.2 kb fragment, containing the coding sequence of the carboxy-terminal end of bovine FXIII along with the TPI1 terminator attached 3' to the bovine Factor XIII stop codon, was isolated.

C. Assembly of pD74

To construct the bovine Factor XIII expression vector, pD16 was cleaved with Bgl II and Xho I. An 11.8 kb fragment was isolated. pD16 is an S. cerevisiae 2-micron plasmid based vector, used to express human Factor XIII, which was derived from pDPOT (ATCC No. 39685) as disclosed in U.S. Patent Application Serial No. 07/525,556, which is incorporated herein by reference. This vector comprises an expression unit including the S. cerevisiae ADH2-4c promoter (published European Patent Application EP 284,044) and a POT1 selectable marker (U.S. Patent No. 4,931,373), which permits plasmid selection in glucose-containing media. The lineralized plasmid was joined in a three-part ligation to the 1.7kb promoter end fragment and the 1.2 kb terminal end fragment of bovine Factor XIII as described above. This construction was designated pD74 (Figure 1).

Plasmid pD74 was used to transform S. cerevisiae host strain, ZM118 (a MATa/MATα diploid homozygous for leu2-3,112 ura3 tpi1::URA3$^+$ bar1 pep4::URA3$^+$ [cir°]). Transformants were selected on synthetic medium lacking tryptophan and supplement with 1M Sorbitol (see Table 1) and maintained on YEPD (0.5% Bacto Yeast Extract, 1% Bacto Peptone and 2% D-Glucose). Factor XIII expression was determined using

mini lysis and fluorometric assay as described in Example V.

Table 1

20 g Glucose
6.7 g Yeast Nitrogen Base without Amino Acid (DIFCO, Detroit, MI)
40 mg adenine
30 mg L-arginine
50 mg L-aspartic acid
20 mg L-histidine free base
60 mg L-isoleucine
80 mg L-leucine
40 mg L-lysine-monohydrochloride
20 mg L-methionine
60 mg L-phenylalanine
50 mg L-serine
50 mg L-tyrosine
40 mg uracil
60 mg L-valine
182.2 g sorbitol
18 g agar (DIFCO)

Mix all ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving, add 150 mg L-threonine. Pour plates and allow to solidify.

Example V

Analysis of bovine the FXIII protein

Cell cultures were pelleted by spinning cells at maximum speed in a clinical centrifuge for 15 minutes. Cell pellets were diluted to 40% wet weight in 1 X lysis buffer (150 mM NaCl, 10 mM B-mercaptoethanol, 5 mM EDTA and 50 mM Tris HCl, pH 7.0). An equal volume of acid washed 0.5 mm leaded glass beads was added to the cell suspension and the sample was vortexed for 1 minute followed by a 1 minute cool

down on ice, repeated five times. The cell-bead suspension was spun at about 1,000 x g for 20 seconds to settle the glass beads. The supernatant was removed to a fresh tube and spun at 14,000 x g for 5 minutes to clarify. The supernatant was removed to a fresh tube and an aliquot was assayed for total protein concentration by the Bradford Method using Protein Assay Reagent 23200 (Pierce Chemical Co., Rockford, IL) as described by the manufacture.

Factor XIIIa content was measured by means of a fluorometric assay. Factor XIII samples were prepared by diluting in 0.05 M Bicine buffer pH 9.0 to a total volume of 200 µl per sample, keeping total protein at 20 µg. Samples were prepared in 10x10x48 mm cuvettes. To each cuvette was added 1.25 ml freshly prepared MDC-Bicine cocktail (0.063 mM monodansylcadaverine (Sigma Chemical Co.) in 0.0 5 M Bicine (N,N-bis[2-hydroxyethyl] glycine; Sigma) pH 9.0, prepared by dissolving 1.34 mg monodansylcadaverine in 0.5 ml of 0.03 M HCl and mixing with an equal volume of 0.1 M Tris-HCl pH 7.4, then combining 0.4 ml of the solution with 24.0 ml of 0.05 M Bicine buffer, pH 9.0) and 50 µl of 0.4 M $CaCl_2$. The solutions were mixed and prewarmed to 37°C for 10 minutes. Fifty microliters of 500 units bovine thrombin was added to each cuvette, the solutions were gently mixed, and the cuvettes were incubated 10 minutes at 37°C. Fifty microliters of freshly prepared 200 mM dithiothreitol was added to each cuvette with gentle mixing. Two hundred microliters 2% N,N-dimethyl casein was added to each cuvette with gentle mixing to begin the assay. Fluorescence excitation at 360 nm and emission at 500 nm using a slit width of 3nm and a water bath temperature of 39°C. The rate of increase in emission was monitored at 500 nm and compared to a Factor XIII standard. Blank was set using 200 µl Bicine buffer in place of Factor XIII. Gain (100%) was set using a 50

μg recombinant Factor XIII standard and omitting stop reagent. Results were compared to the ezymatic rate of a FXIII standard (recombinant human Factor XIII quantitated by amino acid analysis). Factor XIIIa content of samples was determined by assaying samples with and without thrombin. Following gel filtration on Sephacryl S-200, Factor XIIIa content was reduced to approximately 0.3%. Factor XIIIa content of the dissolved lyophilized material was approximately 0.5%.

Results from the activity assay of 20 μg of clarified cell protein are shown in Table 2.

Table 2
fluorometric units/minute

	with thrombin activation	without thrombin activation
recombinant human Factor XIII	19.4	4.2
pD74 bovine Factor XIII	13.9	0.949
vector control	0.0007	not done

These results clearly demonstrate that thrombin-activatable bovine Factor XIII was present.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ZymoGenetics, Inc.
1201 Eastlake Avenue East
Seattle
WA
USA
98102

(ii) TITLE OF INVENTION: Bovine Factor XIII

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ZymoGenetics, Inc.
(B) STREET: 1201 Eastlake Avenue East
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: USA
(F) ZIP: 98102

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Parker, Gary E
(B) REGISTRATION NUMBER: 31-648
(C) REFERENCE/DOCKET NUMBER: 94-18PC

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 206-442-6673
(B) TELEFAX: 206-442-6678

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3876 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 62..2257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAATTCGGCA CGAGGGGCTG GGGCACCTCG GGAGGGAGCG CAGGAACCTG TGAGGCTGAG      60

A ATG TCG GAG TCC TCC GGG ACC GCT TTC GGA GGC AGG AGA GCC ATC          106
  Met Ser Glu Ser Ser Gly Thr Ala Phe Gly Gly Arg Arg Ala Ile
   1               5                  10                  15
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CCC CCC AAC ACC TCC AAT GCA GCA GAG AAC GAC CCC CCC ACC GTG GAG      154
Pro Pro Asn Thr Ser Asn Ala Ala Glu Asn Asp Pro Pro Thr Val Glu
                20                  25                  30

CTG CAG GGC CTG GTG CCC CGG GGC TTC AAC CCA CAA GAC TAC CTT AAT      202
Leu Gln Gly Leu Val Pro Arg Gly Phe Asn Pro Gln Asp Tyr Leu Asn
            35                  40                  45

GTC ACG AAT GTT CAC CTG TTC AAG GAG AGG TGG GAT AGC AAC AAA GTG      250
Val Thr Asn Val His Leu Phe Lys Glu Arg Trp Asp Ser Asn Lys Val
        50                  55                  60

GAT CAC CAC ACC GAC AAA TAC AGC AAC GAC AAG CTG ATC GTT CGT AGA      298
Asp His His Thr Asp Lys Tyr Ser Asn Asp Lys Leu Ile Val Arg Arg
    65                  70                  75

GGA CAG TCT TTC TAC ATT CAG ATT GAC TTC AAT CGT CCC TAT GAC CCC      346
Gly Gln Ser Phe Tyr Ile Gln Ile Asp Phe Asn Arg Pro Tyr Asp Pro
80                  85                  90                  95

ACA AGG GAT CTC TTC AGG GTG GAG TAT GTC ATT GGT CTC TAC CCC CAG      394
Thr Arg Asp Leu Phe Arg Val Glu Tyr Val Ile Gly Leu Tyr Pro Gln
                100                 105                 110

GAG AAT AAG GGA ACC TAC ATT CCA GTC CCT TTG GTC TCT GAG CTG CAG      442
Glu Asn Lys Gly Thr Tyr Ile Pro Val Pro Leu Val Ser Glu Leu Gln
            115                 120                 125

AGT GGC AAG TGG GGG GCG AAG GTG GTC ATG AGA GAG GAC AGG TCT GTC      490
Ser Gly Lys Trp Gly Ala Lys Val Val Met Arg Glu Asp Arg Ser Val
        130                 135                 140

CGG CTG TCT GTC CAG TCT TCT GCA GAC TGC ATT GTG GGG AAG TTC CGC      538
Arg Leu Ser Val Gln Ser Ser Ala Asp Cys Ile Val Gly Lys Phe Arg
    145                 150                 155
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ATG TAC GTG GCT GTC TGG ACC CCC TAT GGG GTC ATC CGC ACC AGC CGA      586
Met Tyr Val Ala Val Trp Thr Pro Tyr Gly Val Ile Arg Thr Ser Arg
160                 165                 170                 175

AAC CCC GAA ACG GAC ACA TAC ATT CTC TTC AAC CCT TGG TGT GAA GAG      634
Asn Pro Glu Thr Asp Thr Tyr Ile Leu Phe Asn Pro Trp Cys Glu Glu
                180                 185                 190

GAT GCT GTG TAC CTG GAA AAT GAA AAA GAA AGA GAA GAG TGC GTC CTG      682
Asp Ala Val Tyr Leu Glu Asn Glu Lys Glu Arg Glu Glu Cys Val Leu
            195                 200                 205

AAT GAC ATC GGG GTT ATT TTT TAT GGA GAC TTC AAC GAC ATC AAG AGC      730
Asn Asp Ile Gly Val Ile Phe Tyr Gly Asp Phe Asn Asp Ile Lys Ser
        210                 215                 220

AGA AGC TGG AGC TAC GGT CAG TTT GAG GAT AGC ATC CTT GAC GCT TGC      778
Arg Ser Trp Ser Tyr Gly Gln Phe Glu Asp Ser Ile Leu Asp Ala Cys
    225                 230                 235

CTG TTT GTG ATG GAC AAA GCG AAT ATG GAC CTT TCC GGC AGA GGG AAT      826
Leu Phe Val Met Asp Lys Ala Asn Met Asp Leu Ser Gly Arg Gly Asn
240                 245                 250                 255

CCC ATC AAA GTC AGC CGT GTT GGG TCT GCC ATG ATC AAT GCC AAG GAC      874
Pro Ile Lys Val Ser Arg Val Gly Ser Ala Met Ile Asn Ala Lys Asp
                260                 265                 270

GAC GAA GGC GTC ATT GCT GGC TCC TGG GAC AAT GTC TAC GCT TAT GGT      922
Asp Glu Gly Val Ile Ala Gly Ser Trp Asp Asn Val Tyr Ala Tyr Gly
            275                 280                 285

GTT CCC CCA TCA GCT TGG ACC GGA AGT GTT GAC ATC CTC CTA GAA TAC      970
Val Pro Pro Ser Ala Trp Thr Gly Ser Val Asp Ile Leu Leu Glu Tyr
        290                 295                 300
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AAG AGT TCT CAG AAA CCA GTC CGC TAT GGT CAG TGC TGG GTT TTT GCT      1018
Lys Ser Ser Gln Lys Pro Val Arg Tyr Gly Gln Cys Trp Val Phe Ala
    305                 310                 315

GGT GTC TTT AAT ACA TTT TTG CGA TGC CTG GGG ATA CCA GCG CGA GTC      1066
Gly Val Phe Asn Thr Phe Leu Arg Cys Leu Gly Ile Pro Ala Arg Val
320                 325                 330                 335

GTC ACC AAC TAT TTC TCA GCC CAT GAC AAT GAT GCC AAC TTG CAA TTG      1114
Val Thr Asn Tyr Phe Ser Ala His Asp Asn Asp Ala Asn Leu Gln Leu
            340                 345                 350

GAC ATA TTC TTG GAA GAA GAC GGG AAC GTG AAC TCC AAA CTC ACC AAG      1162
Asp Ile Phe Leu Glu Glu Asp Gly Asn Val Asn Ser Lys Leu Thr Lys
        355                 360                 365

GAT TCG GTG TGG AAT TAC CAC TGC TGG AAT GAA GCC TGG ATG ACG AGG      1210
Asp Ser Val Trp Asn Tyr His Cys Trp Asn Glu Ala Trp Met Thr Arg
    370                 375                 380

CCG GAC CTT CCC GTT GGG TTT GGA GGT TGG CAA GTC GTG GAC AGC ACC      1258
Pro Asp Leu Pro Val Gly Phe Gly Gly Trp Gln Val Val Asp Ser Thr
385                 390                 395

CCC CAG GAG AAC AGC GAT GGG ATG TAT CGG TGC GGC CCT GCC TCT GTT      1306
Pro Gln Glu Asn Ser Asp Gly Met Tyr Arg Cys Gly Pro Ala Ser Val
400                 405                 410                 415

CAA GCC ATC AAG CAC GGC CAT GTC TGC TTC CAG TTT GAC GCA CCC TTC      1354
Gln Ala Ile Lys His Gly His Val Cys Phe Gln Phe Asp Ala Pro Phe
            420                 425                 430

GTT TTT GCA GAG GTC AAC AGT GAC CTT GTT TAC GTC ACA GCT AAG AAA      1402
Val Phe Ala Glu Val Asn Ser Asp Leu Val Tyr Val Thr Ala Lys Lys
        435                 440                 445
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GAT GGC ACT CAT GTG GTT GAA GCC CTT GAT ACC ACC CAC ATT GGG AAA      1450
Asp Gly Thr His Val Val Glu Ala Leu Asp Thr Thr His Ile Gly Lys
        450                 455                 460

TTA ATC GTG ACC AAA GAA ATT GGA GGA GAT GGC ATG AAG GAC ATC ACG      1498
Leu Ile Val Thr Lys Glu Ile Gly Gly Asp Gly Met Lys Asp Ile Thr
    465                 470                 475

GAC ACC TAC AAA TTC CAG GAA GGT CAA GAA GAA GAG AGG CTG GCC CTG      1546
Asp Thr Tyr Lys Phe Gln Glu Gly Gln Glu Glu Glu Arg Leu Ala Leu
480                 485                 490                 495

GAA ACC GCC ATG ATG TAT GGG GCC AAA AAG GCC CTC AAC ACA GAG GGC      1594
Glu Thr Ala Met Met Tyr Gly Ala Lys Lys Ala Leu Asn Thr Glu Gly
                500                 505                 510

GTC CTC AAA TCG AAG TCT GAT GTC CGC ATG AAC TTC GAG GTG GAG AAC      1642
Val Leu Lys Ser Lys Ser Asp Val Arg Met Asn Phe Glu Val Glu Asn
            515                 520                 525

GCC GTG CTG GGC AGG GAC TTG AAG GTC ATC ATC ACC TTC CGG AAC AAT      1690
Ala Val Leu Gly Arg Asp Leu Lys Val Ile Ile Thr Phe Arg Asn Asn
        530                 535                 540

GGC TCC GCC CGC TAC ACT GTC ACA GCC TAC CTC TCC GGA AAC ATC AGC      1738
Gly Ser Ala Arg Tyr Thr Val Thr Ala Tyr Leu Ser Gly Asn Ile Ser
    545                 550                 555

TTC TAC ACC GGG GTC TCC AAG GCG GAA TTC AAG AAC AAG ACC TCT GAA      1786
Phe Tyr Thr Gly Val Ser Lys Ala Glu Phe Lys Asn Lys Thr Ser Glu
560                 565                 570                 575

GTG ACC CTG GAG CCC TTG TCC TTC AAG AGA GAG GAG GTG CTG ATG GGA      1834
Val Thr Leu Glu Pro Leu Ser Phe Lys Arg Glu Glu Val Leu Met Gly
                580                 585                 590
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GCA GGC GAG TAC ATG GGC CAG CTG CTG GAG CAG GCC TTC CTG CAC TTC      1882
Ala Gly Glu Tyr Met Gly Gln Leu Leu Glu Gln Ala Phe Leu His Phe
            595                 600                 605

TTT GTC ACG GCT CGA GTC AAC GAG ACC AGG GAC GTT CTG GCC AAG CAG      1930
Phe Val Thr Ala Arg Val Asn Glu Thr Arg Asp Val Leu Ala Lys Gln
        610                 615                 620

AAG TCC ATT GCG CTG ACG GTC CCC AAG GTC GTC ATC AAG GTC CGT GGT      1978
Lys Ser Ile Ala Leu Thr Val Pro Lys Val Val Ile Lys Val Arg Gly
    625                 630                 635

GCT CAG GTC GTG GGT TCC AAC ATG GTG GTG ACG GTT GAG TTC ACC AAT      2026
Ala Gln Val Val Gly Ser Asn Met Val Val Thr Val Glu Phe Thr Asn
640                 645                 650                 655

CCT TTA AAA GAG ACG CTT CGC AAT GTC TGG ATC CGC CTG GAT GGT CCT      2074
Pro Leu Lys Glu Thr Leu Arg Asn Val Trp Ile Arg Leu Asp Gly Pro
                660                 665                 670

GGA GTG ACG AAA CCC TTG AGG AAG ATG TTC CGG GAA ATC CGG CCC AAC      2122
Gly Val Thr Lys Pro Leu Arg Lys Met Phe Arg Glu Ile Arg Pro Asn
            675                 680                 685

TCC ACC GTG CAG TGG GAA GAG CTG TGT CGG CCC TGG GTC TCC GGC CCC      2170
Ser Thr Val Gln Trp Glu Glu Leu Cys Arg Pro Trp Val Ser Gly Pro
        690                 695                 700

AGG AAG CTG ATC GCC AGC CTG ACC AGC GAC TCC CTG AGG CAC GTG TAC      2218
Arg Lys Leu Ile Ala Ser Leu Thr Ser Asp Ser Leu Arg His Val Tyr
    705                 710                 715

GGC GAG CTG GAC TTG CAG ATT CAG AGA CGA CCT TCG ATG TAGACGCACG        2267
Gly Glu Leu Asp Leu Gln Ile Gln Arg Arg Pro Ser Met
720                 725                 730

GGGGGCCGAG CTGGACCCAG GCACCTGGCC TCTTGTAGTC TTGGCTGAGG AAGTTCTAAT    2327
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GCAAAAATAG TCAGCTCTTG CTTTAACTTA GCTGTGAAGC CCTGGACAGG ACTGGATAGG 2387

CTCCCAGAGT GGTGACGGCG TGTATTTCAA AGACACGCTT TTCAGTGTGG CTATTCAGTG 2447

CGCAAGGTAG TTTTTAATCA GCCCACCTTC CAAAGGATTC TGAGCATTAG CTTTAATTAA 2507

GCCCTAATTA GGCTCTCGGA GCTCATAAGA GTAAAAGTCA TCATTTATCA TCTCAAATGG 2567

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CACAGATTTA CGTTGTGAGA AGAGGTGGCT CCATATTAAC AAATTGCATT TGCGGGGAAC 2927

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TGGGGATGAG GAATTAACCT CCTTTATTAG TGATTGTACT TCACCTGTCT CCCTGGAAGC 3167

ATCTCTTTGG CACAATGACC CAGGTCCAGG TACAGTTTTA GAGACAGAAT AAACCCAACA 3227

AGTTGGAGAA GCTGGCAGAT TTAGTGACCA GATGTGGAAG GGCAGCCACT ACTTCTCTCA 3287

TGCTTCACAT CCCCCATGTT GAGACCTCAG CTCAGCACAC AAGTGCTAGA AGCTGAAACA 3347

GACTCCACCC TGCAAACAGC CAGTGGGACT GCACAGCCGA TGGCAGAGGA CATGGATATC 3407

ACTGGAATTC GGCTCTAAGG TTCCAACAGG CAAGGCGACC AAATATTTAT CTGCAAGGCT 3467

GATTTTTTTG TCCAAATTAC CAAACCGATA TGCCTAGAGT ATGATTTAGG TCGGTAAATT 3527

GTGCTTCTTA GCAGAAGAAA GGAAAGACGA ATAGTGAGGA GGAAGCAGGG GGAACGCCAG 3587

AATGGAATTG TGTGTGGTCT CTACAACCAC ATTTCTAGGC CTTTGAGACG GCTCCTGAGC 3647

CTTCGGCACT GGAATCCATG AGGGTTAGCC AGTCCCCTTC ACAGACGCCA CGTACCTAAC 3707

TCTACTAAGT AATCCCCCAG CATTTGCCAA GGCTTCCAAT GCTCAGTTCT AAAATGAAAT 3767

GCATTTTGCT GGACTGTTAA ACCGGCTTAC TGTAGTATAT TCTTATTAAC TAGAATGTAA 3827

TCAAAGCTTA AAATAAAGCT AATCTGATTG TAAAAAAAAA CGGCACGAG 3876

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 732 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Glu Ser Ser Gly Thr Ala Phe Gly Gly Arg Arg Ala Ile Pro
1 5 10 15

Pro Asn Thr Ser Asn Ala Ala Glu Asn Asp Pro Pro Thr Val Glu Leu
20 25 30

Gln Gly Leu Val Pro Arg Gly Phe Asn Pro Gln Asp Tyr Leu Asn Val
35 40 45

Thr Asn Val His Leu Phe Lys Glu Arg Trp Asp Ser Asn Lys Val Asp
50 55 60

His His Thr Asp Lys Tyr Ser Asn Asp Lys Leu Ile Val Arg Arg Gly
65 70 75 80

Gln Ser Phe Tyr Ile Gln Ile Asp Phe Asn Arg Pro Tyr Asp Pro Thr
85 90 95

Arg Asp Leu Phe Arg Val Glu Tyr Val Ile Gly Leu Tyr Pro Gln Glu
100 105 110

Asn Lys Gly Thr Tyr Ile Pro Val Pro Leu Val Ser Glu Leu Gln Ser
115 120 125

Gly Lys Trp Gly Ala Lys Val Val Met Arg Glu Asp Arg Ser Val Arg
130 135 140

Leu Ser Val Gln Ser Ser Ala Asp Cys Ile Val Gly Lys Phe Arg Met
145 150 155 160

Tyr Val Ala Val Trp Thr Pro Tyr Gly Val Ile Arg Thr Ser Arg Asn
165 170 175

Pro Glu Thr Asp Thr Tyr Ile Leu Phe Asn Pro Trp Cys Glu Glu Asp
180 185 190

Ala Val Tyr Leu Glu Asn Glu Lys Glu Arg Glu Glu Cys Val Leu Asn
195 200 205

Asp Ile Gly Val Ile Phe Tyr Gly Asp Phe Asn Asp Ile Lys Ser Arg
210 215 220

Ser Trp Ser Tyr Gly Gln Phe Glu Asp Ser Ile Leu Asp Ala Cys Leu
225 230 235 240

Phe Val Met Asp Lys Ala Asn Met Asp Leu Ser Gly Arg Gly Asn Pro
245 250 255

Ile Lys Val Ser Arg Val Gly Ser Ala Met Ile Asn Ala Lys Asp Asp
260 265 270

Glu Gly Val Ile Ala Gly Ser Trp Asp Asn Val Tyr Ala Tyr Gly Val
275 280 285

Pro Pro Ser Ala Trp Thr Gly Ser Val Asp Ile Leu Leu Glu Tyr Lys
290 295 300

Ser Ser Gln Lys Pro Val Arg Tyr Gly Gln Cys Trp Val Phe Ala Gly
305 310 315 320

Val Phe Asn Thr Phe Leu Arg Cys Leu Gly Ile Pro Ala Arg Val Val
325 330 335

Thr Asn Tyr Phe Ser Ala His Asp Asn Asp Ala Asn Leu Gln Leu Asp
340 345 350

Ile Phe Leu Glu Glu Asp Gly Asn Val Asn Ser Lys Leu Thr Lys Asp
355 360 365

Ser Val Trp Asn Tyr His Cys Trp Asn Glu Ala Trp Met Thr Arg Pro
370 375 380

Asp Leu Pro Val Gly Phe Gly Gly Trp Gln Val Val Asp Ser Thr Pro
385 390 395 400

Gln Glu Asn Ser Asp Gly Met Tyr Arg Cys Gly Pro Ala Ser Val Gln
405 410 415

Ala Ile Lys His Gly His Val Cys Phe Gln Phe Asp Ala Pro Phe Val
420 425 430

Phe Ala Glu Val Asn Ser Asp Leu Val Tyr Val Thr Ala Lys Lys Asp
435 440 445

Gly Thr His Val Val Glu Ala Leu Asp Thr Thr His Ile Gly Lys Leu
450 455 460

Ile Val Thr Lys Glu Ile Gly Gly Asp Gly Met Lys Asp Ile Thr Asp
465 470 475 480

Thr Tyr Lys Phe Gln Glu Gly Gln Glu Glu Glu Arg Leu Ala Leu Glu
485 490 495

Thr Ala Met Met Tyr Gly Ala Lys Lys Ala Leu Asn Thr Glu Gly Val
500 505 510

Leu Lys Ser Lys Ser Asp Val Arg Met Asn Phe Glu Val Glu Asn Ala
515 520 525

Val Leu Gly Arg Asp Leu Lys Val Ile Ile Thr Phe Arg Asn Asn Gly
530 535 540

Ser Ala Arg Tyr Thr Val Thr Ala Tyr Leu Ser Gly Asn Ile Ser Phe
545 550 555 560

Tyr Thr Gly Val Ser Lys Ala Glu Phe Lys Asn Lys Thr Ser Glu Val
565 570 575

Thr Leu Glu Pro Leu Ser Phe Lys Arg Glu Glu Val Leu Met Gly Ala
580 585 590

Gly Glu Tyr Met Gly Gln Leu Leu Glu Gln Ala Phe Leu His Phe Phe
595 600 605

Val Thr Ala Arg Val Asn Glu Thr Arg Asp Val Leu Ala Lys Gln Lys
610 615 620

Ser Ile Ala Leu Thr Val Pro Lys Val Val Ile Lys Val Arg Gly Ala
625 630 635 640

Gln Val Val Gly Ser Asn Met Val Val Thr Val Glu Phe Thr Asn Pro
645 650 655

Leu Lys Glu Thr Leu Arg Asn Val Trp Ile Arg Leu Asp Gly Pro Gly
660 665 670

Val Thr Lys Pro Leu Arg Lys Met Phe Arg Glu Ile Arg Pro Asn Ser
675 680 685

Thr Val Gln Trp Glu Glu Leu Cys Arg Pro Trp Val Ser Gly Pro Arg
690 695 700

Lys Leu Ile Ala Ser Leu Thr Ser Asp Ser Leu Arg His Val Tyr Gly
705 710 715 720

Glu Leu Asp Leu Gln Ile Gln Arg Arg Pro Ser Met
725 730

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC667

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATAGAATATC AAGCTACA 18

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2045

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATATAAAGA AAAGAAG 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC6091

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GAGCACAGAA TTCACTACTC GAGGCGGCCG CTTTTTTTTT TTTTTTTTT 49

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC218

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCTGCCTGC CGAAC 15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC219

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACTAGGCAGG CTAGGTCACA GCCC 24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC7386

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GATCCAGATC TCACAATGTC GGAGTCCTCC GGGACCGCTT TCGGAGGCAG GAGAGCCATC 60

CCCCCCAA 68

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC7396

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAGGTGTTGG GGGGGATGGC TCTCCTGCCT CCGAAAGCGG TCCCGGAGGA CTCCGACATT 60

GTGAGATCTG 70

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC7389

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CACCTCCAAT GCAGCAGAGA ACGACCCCCC CACCGTGGAG CTGCAGGGCC TGGTGCC 57

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC7382

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCGGGGCACC AGGCCCTGCA GCTCCACGGT GGGGGGGTCG TTCTCTGCTG CATTG 55

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC7384

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGTGTACGGC GAGCTGGACT TGCAGATTCA GAGACGACCT TCGATGTAGT 50

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC7383

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTAGACTACA TCGAAGGTCG TCTCTGAATC TGCAAGTCCA GCTCGCCGTA 50

Claims:

1. An isolated DNA molecule encoding a bovine Factor XIII selected from the group consisting of:

a) DNA molecules comprising a coding sequence as shown in SEQ ID NO: 1 from nucleotide 62 to nucleotide 2257;

b) DNA molecules complementary to (a);

c) allelic variants of (a) or (b); and

d) DNA molecules that encode for the protein shown in SEQ ID NO: 2.

2. The isolated DNA molecule of claim 1, wherein the DNA molecule encodes for the protein shown in SEQ ID NO: 2.

3. A DNA construct for the expression of bovine Factor XIII, which comprises the following operably linked elements:

a transcriptional promoter;

a DNA segment selected from the group consisting of:

a) DNA molecules comprising a coding sequence as shown in SEQ ID NO: 1 from nucleotide 62 to nucleotide 2257;

b) DNA molecules complementary to (a);

c) allelic variants of (a) or (b); and

d) DNA molecules that encode for the protein shown in SEQ ID NO: 2; and

a transcriptional terminator.

4. A DNA construct according to claim 3, wherein the DNA molecule encodes for the protein shown in SEQ ID NO: 2.

5. An isolated bovine Factor XIII polypeptide comprising the amino acid sequence of SEQ ID NO: 2 from amino acid residue 2 to amino acid residue 732.

6. A cultured cell transfected or transformed with the DNA construct of claim 3.

7. The cultured cell of claim 6, wherein said cell is a yeast cell or a mammalian cell.

8. A method of producing bovine Factor XIII which comprises culturing a cell transformed or transfected with the DNA construct of claim 3, and isolating the Factor XIII from the cells.

9. The method of claim 8, wherein said cells are yeast cells.

10. A method for increasing the water binding capacity of a protein comprising:

mixing a protein that contains a substrate with a bovine Factor XIII of claim 5, wherein the substrate is crosslinkable by Factor XIII, to provide a mixture; and

incubating the mixture for a period of time sufficient for the bovine Factor XIII to react with the substrate, thereby increasing the water binding capacity of the protein.

11. The method of claim 10, wherein the protein is selected from the group consisting of casein, caseinate, whey protein, soy protein, blood plasma, fish protein, wheat protein, maize protein, egg albumin, rape seed protein and potato protein.

12. A method of producing a food product with increased water binding capacity comprising:

mixing a food product that contains a protein containing a substrate with a bovine Factor XIII of claim 5, wherein the substrate is crosslinkable by Factor XIII, to provide a mixture; and

incubating the mixture for a period of time sufficient for the bovine Factor XIII to react with the substrate, thereby increasing the water binding capacity of the food product.

13. The method of claim 12, wherein the food product is selected from the group consisting of milk and meat from beef, pork, poultry or fish.

14. The method of claim 12, wherein the food product comprises a mixture of ingredients, wherein one of the ingredients is a protein selected from the group consisting of casein, caseinate, whey protein, soy protein, blood plasma, fish protein, wheat protein, maize protein, egg albumin, rape seed protein and potato protein.

15. A method of modifying the amino acid composition of a protein comprising:

mixing a protein containing a substrate with a bovine Factor XIII of claim 5 and an amino acid,

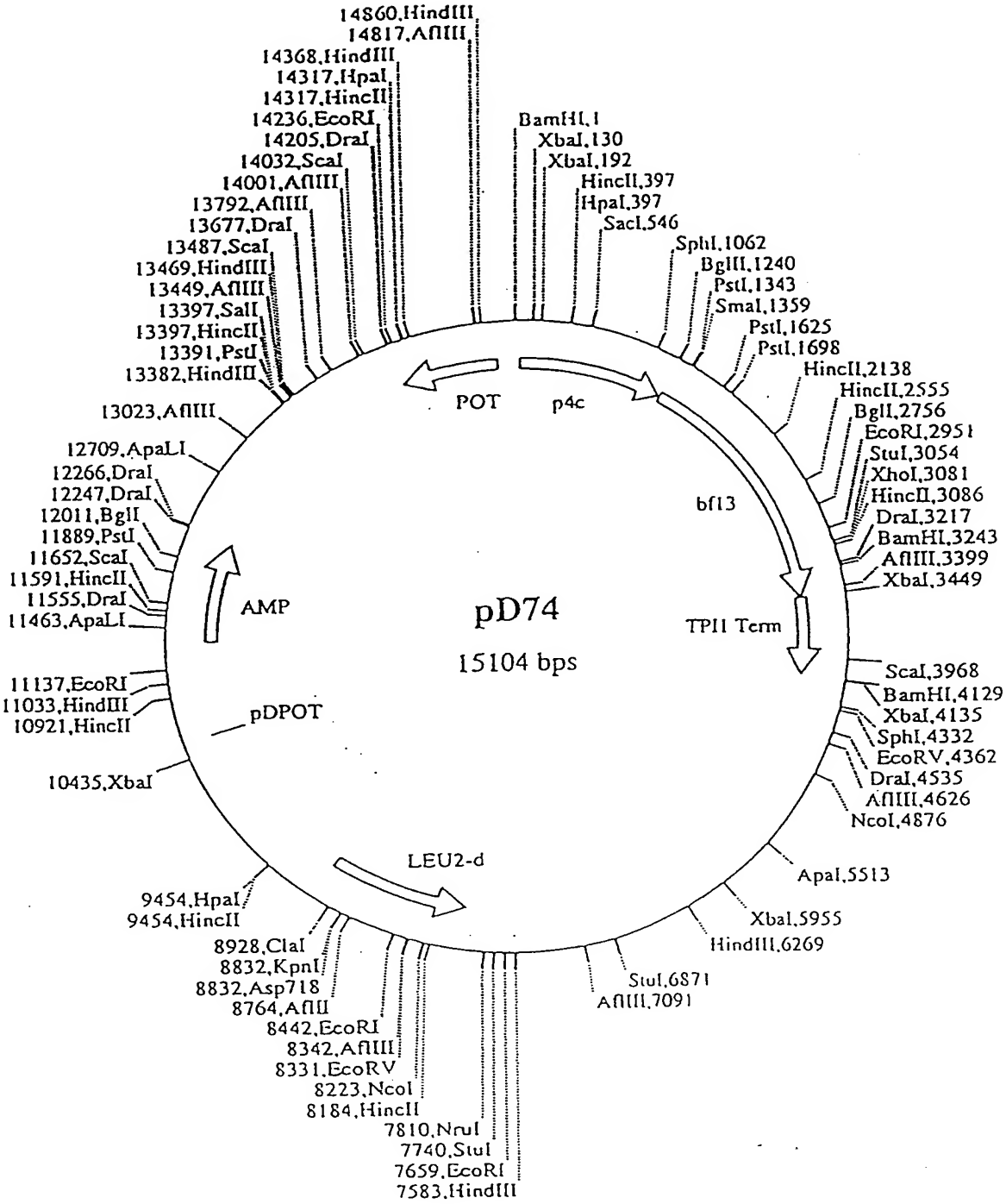
wherein the substrate is crosslinkable by a bovine Factor XIII, to provide a mixture; and

reacting the mixture for a period of time sufficient to covalently bind the amino acid to the protein.

16. A method of binding a first protein to a surface of an insoluble second protein comprising:

reacting a first protein and a bovine Factor XIII of claim 5 with a second, insoluble protein comprising a substrate that is crosslinkable by Factor XIII, for a time sufficient to result in a crosslinked complex of the first protein bound to the surface of the second protein.

1/1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LEU2-d
14860,HindIII
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14368,HindIII
14317,HpaI
14317,HincII
14236,EcoRI
14205,DraI
14032,ScaI
14001,AflIII
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13677,DraI
13487,ScaI
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13449,AflIII
13397,SalI
13397,HincII
13391,PstI
13382,HindIII
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12266,DraI
12247,DraI
12011,BglI
11889,PstI
11652,ScaI
11591,HincII
11555,DraI
11463,ApaLI
11137,EcoRI
11033,HindIII
10921,HincII
10435,XbaI
9454,HpaI
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8764,AflII
8442,EcoRI
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8331,EcoRV
8223,NcoI
8184,HincII
7810,NruI
7740,StuI
7659,EcoRI
7583,HindIII
BamHI,1
XbaI,130
XbaI,192
HincII,397
HpaI,397
SacI,546
SphI,1062
BglII,1240
PstI,1343
SmaI,1359
PstI,1625
PstI,1698
HincII,2138
HincII,2555
BglI,2756
EcoRI,2951
StuI,3054
XhoI,3081
HincII,3086
DraI,3217
BamHI,3243
AflIII,3399
XbaI,3449
ScaI,3968
BamHI,4129
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EcoRV,4362
DraI,4535
AflIII,4626
NcoI,4876
ApaI,5513
XbaI,5955
HindIII,6269
StuI,6871
AflIII,7091

Figure

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 95/17026

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C07K14/745 C12N1/21 C12N5/10 A61K38/36
C12P21/00 C12N9/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMISTRY, vol. 25, 1986, pages 6900-6906, XP002003522 A. ICHINOSE ET AL.: "Amino acid sequence of the a Subunit of human FXIII" *see the whole document* ---	1-16
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 83, 1986, pages 8024-8028, XP002003523 U. GRUNDMANN ET AL.: "Characterization of the cDNA coding for human FXIIIa" *see the whole article* ---	1-16
Y	EP,A,0 268 772 (ZYMOGENETICS INC.) 1 June 1988 *see the whole document* --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 May 1996	17-06-96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Marie, A

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INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/US 95/17026

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 494 702 (BEHRINGWERKE AG.) 15 July 1992 *see the whole document* ---	1-16
Y	J. AGRIC. FOOD CHEM., vol. 40, 1990, pages 2052-2056, XP002003524 C. DE BACKER.ROYER ET AL.: "Polymerisation of meat and soyabean proteins by human placental calcium-activated FXIII" *see the whole document* ---	1-16
Y	J. AGRIC. FOOD CHEM., vol. 41, 1992, pages 2208-2214, XP002003525 M.-C. ALEXANDRE ET AL.: "Wheart gamma gliadin as substrate for bovine plasma FXIII" *see the whole document* ---	1-16
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PCT/US 95/17026

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